

cellular resources, affecting expression of the module of interest (resource-coupled module). The module of interest also applies a load to the resources. FIG. 1B: An incoherent feedforward loop (iFFL) device within the module of interest decouples the module's output from resource variability. An endoribonuclease (endoRNase/ERN) produced by the identical promoter as the output represses the output by binding to a specific target site in its 5'UTR and cutting the mRNA. FIG. 1C: A simplified schematic of the iFFL showing how cellular resources directly contribute to the production of both the endoRNase and the output. FIG. 1D: The expected behavior of the output of the resource-coupled and resource-decoupled modules in response to resource loading by other modules. FIG. 1E: Experimental model system of resource loading. The module of interest comprises a constitutively expressed protein (Output₁). In a competitor module, Gal4 transcriptional activators (TAs) drive expression of another protein (Output₂). Different activation domains (ADs) were fused to the DNA binding domain (DBD) of Gal4. A reporter (Gal4 Marker) was titrated together with the Gal4 TAs to mark their delivery per cell. Note that the transfection marker (TX Marker) also competes for resources, but that process is omitted from schematic for simplicity. FIG. 1F: Dose-dependent effect of Gal4 TAs on Outputs. The markers indicate median expression levels from three experimental repeats. The lines represent fits of the steady-state resource competition model. The CV(RMSE) is the root-mean-square error between the model and data, normalized by the mean of the data. All data were measured by flow cytometry at 48 hours post-transfection in HEK-293FT cells. FIG. 1G: Gene expression is affected by many factors that reflect cell state. These factors include resource availability, the presence of off-target interactors, and (for ectopic systems) varying gene dosage (copy number). These factors as disturbances can be modeled to be rejected with an iFFL. FIG. 1H: iFFLs are a class of network structure wherein an upstream node both activates and represses a downstream node through divergent branches. iFFLs controllers can provide robust perfect adaptation (RPA) of output expression to upstream disturbances. Illustrated are designs previously published using transcription factors (TFs) and microRNAs (miRNAs, miRs), as well as the new design using endoRNases. Strengths/weaknesses of each design are compared in the table to the right. Notably, the condition for non-cooperativity in TF-promoter binding hampers scalability of TF iFFLs.

[0017] FIGS. 2A-2F: Effect of resource competition between promoters and activators across cell lines. FIG. 2A: Genetic model system to study competition for resources between different combinations of promoters, Gal4 transcriptional activators (TAs), and cell lines. The specific promoters, activation domains (ADs) fused to Gal4, and cell lines are shown alongside the data in FIGs. 2B-2. FIG. 2B: Nominal Outputs are the median expression levels of each promoter in Module 1 ($\{P\}$:Output1) in each cell line when co-transfected with Gal4-None (i.e. the Gal4 DNA binding domain), which does not load resources. FIG. 2C: Fold-changes (Fold- Δ s) in the level of $\{P\}$:Output1 in response to Gal4 TAs. The Fold- Δ s are computed independently for each promoter and cell line by dividing the median level of $\{P\}$:Output1 for each sample co-transfected with different Gal4 TAs by the Nominal Output. FIG. 2D: The five promoter-activator combinations in each cell line with the smallest effect or largest negative effect on the level of

Output1. The plots show the mean and standard deviation of three experimental repeats (represented by the individual points). FIG. 2E: Specificity of EndoRNase cutting. Various endoRNases were tested for specificity in gene knockdown. Each endoRNase (rows) was co-transfected in HEK-293FT cells with a reporter gene expressing a fluorescent protein with a target site (XXXr or XXXR, columns) in the 5'UTR of the output mRNA. The heatmap shows expression levels normalized by a transfection marker. Brightness corresponds to log-transformed output levels measured with flow cytometry. FIG. 2F: Target Site Placement. CasE EndoRNase and miR-FF4 iFFLs were tested with target sites in either the 5' or 3' UTR of the output mRNA. Circuit variants were co-transfected into HEK-293FT cells with a constitutive transfection marker. Adaptation of output expression to the DNA copy number was assessed by measuring the output and transfection marker by flow cytometry. The results show that adaptation to copy number only occurs when the miRNA or endoRNase are targeted to the 5'UTR. $CV(RMSE)=RMSE/\text{mean}(y)$. All data were measured by flow cytometry at 48 hours post-transfection in the cell lines indicated. FIGS. 2B-2C show the geometric mean of median measurements and mean of fold-changes from three experimental repeats, respectively.

[0018] FIGS. 3A-3E: Adaptation to resource perturbations of an endoRNase-based iFFL module. FIG. 3A: A schematic of the iFFL module. The expression of the output gene (y) may change due to variations in the availability of free transcriptional (TX) and translational (TL) resources (R). When the gene is regulated by an endoRNase (x), an unintended increase (decrease) in R increases (decreases) the amount of endoRNase to reduce (increase) the amount of the output by enhancing its mRNA degradation. This action compensates for the unintended increase (decrease) in the regulated gene's production rate due to variations in R . Since the same pool of TX and TL resources is also used to express the transfection marker z in a transient transfection experiment, the marker's concentration z was used as a proxy to quantify R experimentally. FIG. 3B: The steady state output level (y) of the iFFL can be written as a function of the marker level (z). The performance of an iFFL was evaluated by (i) its maximum output (Y_{\max}) and (ii) its robustness to variation in R and therefore z , characterized by (Z_{50}). In this model, both Y_{\max} and Z_{50} are linear functions of ϵ , which can be used as a design parameter (see equation (2)). ϵ is proportional to the decay rates of the endoRNase and output mRNA and is inversely proportional to the production rate and catalytic efficiency of the endoRNase. FIG. 3C: An increase in the number of uORFs in the 5' UTR of the endoRNase's transcript leads to a decrease in its TL initiation rate. It was modeled as an increase in the dissociation constant between the ribosome and the endoRNase's mRNA transcript (kx), which increases ϵ . The relationship between the number of uORFs and the fold decrease in TL initiation (i.e., parameter kx in the model) is summarized in the table using previously-published experimental data³⁸. FIG. 3D: Sample experimental data corresponding to the theoretical plot in b. n indicates the number of uORFs in the 5'UTR of CasE. The shroud indicates the 5th to 95th percentiles of the output in each half-log-decade TX Marker bin. The thin lines mark the 25th and 75th percentiles, and the thick line marks the median Output in each bin. FIG. 3E: Comparison between experimentally measured inverse robustness metric (Z_{50}) and maximum